Mitochondrial dynamics, mitophagy and biogenesis in neonatal hypoxic-ischaemic brain injury

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Keywords

Mitochondria, neonatal, fission, fusion, mitophagy, biogenesis, brain injury

Abbreviations

•OH^-; hydroxyl radicals
AIF; apoptosis inducing factor
AMPA; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP; adenosine triphosphate
Bax; Bcl-2 associated X protein
Bak; Bcl-2 antagonist/killer
Bcl-2; B-cell lymphoma 2
Bcl-xL; B-cell lymphoma extra large
tBid; truncated BH3 interacting domain protein
Bim; Bcl-2-like protein 11
BNIP3; BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
Cdk1; cyclin dependent kinase 1
CHCHD4; Coiled coil helix-Coiled coil helix domain containing protein 4
Cyt c; cytochrome c
DAMP; Damage associated molecular pattern
DRP1; Dynamin related protein 1
EPO; Erythropoietin
ER; endoplasmic reticulum
ERR; Estrogen-related receptor
Fis1; fission protein 1
FUNDC1; FUN14 Domain Containing 1
H_2O_2; hydrogen peroxide
HI; hypoxia-ischemia
HIE; hypoxic-ischemic encephalopathy
HIF-1α; hypoxia-inducible factor-1 α
IMM; inner mitochondrial membrane
LC3B; microtubule-associated protein 1A/1B light chain 3B
LPS; lipopolysaccharide
MCAO; middle cerebral artery occlusion
MCL-1; myeloid cell leukaemia sequence 1
Mff; mitochondrial fission factor
Mfn1/2; mitofusin 1/2
MiD49/51; mitochondrial dynamics proteins of 49 and 51 kDa
MOMP; mitochondrial outer membrane permeabilisation
mtDNA; mitochondrial DNA
NADH; Nicotinamide adenine dinucleotide
NFAT; Nuclear factor of activated T-cell
Nix; NIP3-like protein X
NLR; Nod-like receptor
NLRP3; Nod-like receptor protein 3
NO; Nitric oxide
NOS; nitric oxide synthase
NR2B; NMDA receptor 2B
NRF1/2; nuclear response factor 1/2
$O_2^{\bullet-}$; Superoxide
OGD; oxygen glucose deprivation
OMM; outer mitochondrial membrane

ONOO-; peroxynitrite
OPA1; optic atrophy protein 1
OXPHOS; oxidative phosphorylation
PGC-1α; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PINK1; PTEN-induced putative kinase 1
PKA; protein kinase A
PPAR; Peroxisome proliferator-activated receptor
RLR; Rig-like receptor
RNS; reactive nitrogen species
ROS; reactive oxygen species
SOD; superoxide dismutase
TCA; tricarboxylic acid cycle
Tfam; Mitochondrial transcription factor A
TLR; toll like receptor
TNFα; Tumour necrosis factor-alpha
TPP; triphenylphosphonium
TRAIL; TNF-related apoptosis-inducing ligand
WT; wild type

Abstract

Hypoxic-ischaemic encephalopathy, resulting from asphyxia during birth, affects 2-3 in every 1000 term infants and depending on severity, brings about life-changing neurological consequences or death. This hypoxic-ischaemia (HI) results in a delayed neural energy failure during which the majority of brain injury occurs. Currently, there are limited treatment options and additional therapies are urgently required. Mitochondrial dysfunction acts as a focal point in injury development in the immature brain. Not only do mitochondria become permeabilised, but recent findings implicate perturbations in mitochondrial dynamics (fission, fusion), mitophagy and biogenesis. Mitoprotective therapies may therefore offer a new avenue of intervention for babies who suffer life-long disabilities due to birth asphyxia.

Introduction

Moderate to severe hypoxic-ischaemic encephalopathy (HIE), caused by birth asphyxia in term or near-term babies, affects 1-2 in every 1000 live births in the UK and far more in the developing world [1-3]. The consequences for babies and parents affected by HIE are devastating; 15-20% of infants will die in the postnatal period and a further 25% will develop severe and long-lasting neurological impairments presenting significant emotional and financial burdens [4]. Currently, therapeutic hypothermia is the only mandated therapy approved for term HIE which, when initiated within the first 6h of birth, doubles the chance of survival without neurocognitive defects [5-8]. However, the number needed to treat for an improvement is 7 – 8 infants [5, 7] highlighting the urgent need for synergistic, additive treatments. Crucially, though, therapeutic hypothermia provides proof-of-concept for neuroprotective intervention *post-injury* and represents the first evidence-based therapy for term HI [5-7, 9-13].

We and others have shown that HI triggers a broad spectrum of signalling events [14-17] culminating in cell death characterized morphologically by a mixed necrotic/necroptotic-apoptotic phenotype [15, 18-20]. However, data from our lab and others strongly suggest that mitochondrial dysfunction acts as a hub for these diverse injury responses [18, 21, 22]. Mitochondrial permeabilisation, electron transport impairment reducing ATP generation, production of ROS, apoptosis and cell death are all key elements of the evolution of injury in the neonatal brain, and both mitochondria and anti-oxidant defence systems are more vulnerable in the neonatal brain than the adult brain [21]. More recently, perturbation of mitochondrial dynamics (fission, fusion) and mitophagy have also been described in the immature brain after insult. Therefore interventions designed to protect mitochondrial function in the hours following hypoxia-ischemia should thus safeguard neuronal survival, providing additional neuroprotection for infants where hypothermia alone is inadequate.

Mitochondria act as hubs for development of brain injury

Phases of neonatal brain injury

Clinical MRI studies report that encephalopathy following perinatal asphyxia and/or hypoxia-ischaemia (HI) in the term neonate is characterized predominantly by lesions in grey matter structures such as the thalamus and basal ganglia, additional cortical abnormalities and associated white matter [23-25]. In near-term and term neonates (>36 weeks) it is predominantly neurons that are most vulnerable to injury and death, with the extent of lesion size being strongly associated with the severity of long-term impairments [26]. During the primary phase of injury, restricted cerebral blood

flow reduces oxygen and glucose delivery causing a switch to anaerobic respiration, reducing phosphocreatine and ATP, and increasing lactic acid production [27, 28]. Reperfusion enables a latent phase to occur and there is a transient recovery of ATP reserves to almost physiological levels providing a treatment window between 1 and 6 hours following injury [29]. However, a secondary phase of rapid energy failure follows [30-32] which lasts from hours to days, during which neuronal cell death occurs. Numerous rodent studies demonstrate that preventing this delayed cell death phase offers significant long-term neuroprotection [33-36]. If intervention is unsuccessful, a tertiary phase of injury is initiated which lasts from weeks to years, and which is dependent on persistent inflammation and epigenetic modifications [37].

Mitochondrial dysfunction in HIE

The molecular consequences of HI injury are many and varied, but a common target of this metabolic dysfunction is mitochondrial impairment [21]. The initial depletion of high energy phosphates results in membrane transporter deficits leading to accumulation of sodium and calcium within the cell, subsequent depolarisation and release of excitotoxic levels of glutamate [14, 38]. The activation of glutamatergic AMPA and NMDA receptors perpetuates the influx of calcium into the neuron which accumulates within the endoplasmic reticulum and the mitochondrial matrix (Figure 1). Mitochondria in the immature brain are less efficient at buffering calcium and this influx leads to mitochondrial swelling within 30 min – 3h of the original insult, triggering calcium-activated proteases and lipases [15]. Calmodulin-dependent activation of nitric oxide synthase generates nitric oxide which in turn results in the blockage of mitochondrial respiratory complex IV and formation of peroxynitrites [39]. Additionally, although the direct mechanisms of mitochondrial calcium-mediated ROS production are still unclear [40], calcium overload coincides with upregulated superoxide production due to electron leakage from complexes I and III of the electron transport chain (Figure 1). The accumulation of hydroxyl and peroxynitrite radicals leads to lipid peroxidation, protein nitrosylation and DNA damage which is more acute in the immature brain given its limited antioxidant capacity and free availability of catalytic iron [39].

Mitochondria are also susceptible to permeabilisation after neonatal HI injury [41]. In the immature brain, this has been identified as mitochondrial outer membrane permeabilisation (MOMP; Figure 1) in contrast to formation of the mitochondrial permeability transition pore where both inner and outer membranes are opened [42]. MOMP is mediated by the balance of pro- and anti-apoptotic members of the Bcl-2 family. When anti-apoptotic proteins Bcl-2, Bcl-xL and MCL1 lift their inhibition of proapoptotic Bax it becomes activated and translocates to the outer mitochondrial membrane (OMM) where it oligomerises and forms pores [43]. Similarly, mitochondrial pores can be formed with another family member, Bak, already localised at the OMM [44]. HI-mediated activation of upstream enzymes

such as caspase-2 and p53 can also target proapoptotic Bcl-2 proteins such as Bid, Noxa and PUMA resulting in Bax-mediated MOMP (Figure 1; [35, 45, 46]). We and others have shown that pharmacological or genetic inhibition of p53, Bax and caspase-2 results in attenuation of the injury process and a reduction in infarct volume in a well characterised rodent model of neonatal HI [35, 36, 45, 47-49]. In particular, brain injury is significantly reduced in Bax knockout mice or after treatment with Bax inhibitory peptide reaffirming that in the immature brain, Bax-dependent MOMP plays a critical role in neuronal cell death [36, 42].

Once permeabilised, components of the apoptotic cascade are leaked into the cytosol including cytochrome c, apoptosis-inducing factor (AIF), endonuclease G and SMAC/Diablo [50]. Cytosolic cytochrome c and Apaf-1 combine to form the apoptosome, recruiting and activating caspase-9 and subsequently activating caspase-3, a process enhanced by SMAC/Diablo (Figure 1). Active caspase-3 cleaves Caspase-activated DNase (CAD) inhibitors thus leading to CAD activation and chromatin fragmentation occurs [44]. In parallel, caspase-independent cell death is also triggered through the mitochondrial release of AIF and Endo G which translocate to the nucleus and result in chromatin fragmentation (Figure 1). It is worth noting that due to rapid brain development, caspase-dependent and independent mechanisms are more critical in the immature brain than the adult brain [51, 52] and expression of key proteins is markedly upregulated. Furthermore recent studies have identified new regulators of these apoptotic pathways which are specifically perturbed after neonatal HI injury in a rodent model. CHCHD4 is an AIF-interacting protein implicated in protein import and Ca^{2+} uptake. Genetic CHCHD4 haploinsufficiency resulted in reduced infarct size and a reduction in caspase-independent neuronal cell death [53]. Severe HI insult *in vivo* also results in the loss of the AIF negative regulatory protein Iduna, exacerbating the injury as well as contributing to the development of neurocognitive impairment [54, 55].

Mitochondria in perinatal inflammation

Inflammation is an important risk factor for injury in the developing brain [56]. Mitochondria act as central hubs in the innate immune system by regulating receptors, including RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and Toll-like receptors (TLR) [57]. As mitochondria are the nexus of metabolic pathways, current evidence supports the hypothesis that mitochondrial metabolism controls functions and fate of immune cells [58], including regulation of cytokine or chemokine responses [59, 60]. Our recent data shows that inflammation-induced suppression of mitochondrial respiration may contribute to increased susceptibility to HI injury in the neonatal brain [61]. However,

the connection between mitochondrial function, inflammation, and energy metabolism in cerebral immune cells is not well characterized.

Microglia, the immune cells of the brain, orchestrate the inflammatory response to diverse insults, and these cells can have both beneficial and detrimental effects on the brain that may be linked to mitochondrial function. A short exposure of a low dose of lipopolysaccharide (LPS) causes a transient increase in oxidative phosphorylation (OXPHOS) in microglia, whereas a prolonged exposure to high dose LPS causes suppression of OXPHOS and mitochondrial respiration. The suppression of OXPHOS forces a shift in metabolism towards glycolysis to meet cellular energy demand and an increased pro-inflammatory cytokine profile. Other mechanisms by which mitochondria influence immune cell fate decisions include mitochondrial ROS and generation of TCA cycle metabolites that control epigenetic modifications. Mitochondrial ROS diffuse into the cytoplasm and modulate transcription factors, such as hypoxia-inducible factor 1α (HIF1α), nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB), promoting inflammatory gene expression [62]. Transcriptional changes also lead to altered TCA cycle metabolites such as α-ketoglutarate and succinate, which are substrates and products of histone demethylases [63]. Similarly, sirtuin lysine deacetylases responding to the ratio of NAD/NADH have been implicated in the regulation of metabolism by histone demethylation [64].

Injured mitochondria release extracellular mitochondrial DNA (mtDNA), which can act as damage-associated molecular pattern (DAMP) molecules and induce inflammation in response to non-infectious injury [65]. It has been reported that DAMPs serve as 'Signal 0s' that binds to innate immune receptors to promote autophagy termed 'immunophagy' [66, 67]. Mounting evidence shows that selective autophagy or mitophagy regulates innate immune responses primarily by maintaining a functional cohort of mitochondria within the cell to prevent damaging levels of activation [68]. Oxidized mtDNA released into the cytosol binds to NLRP3 resulting in activation and caspase-1 maturation [69], which in turn can induce an inflammatory form of cell death referred to as pyroptosis and disruption of glycolytic flux [70, 71]. Further, when released from necrotic cells, mitochondrial transcription factor A (Tfam) can also act as a specific DAMP causing pro-inflammatory and cytotoxic responses through lysosomal dysfunction in T cells [72] [73].

Mitochondrial Dynamics

Although evidence implicating mitochondria as the hub of the injury response after HI has centred on identification of the mechanisms leading to MOMP [21], recent studies suggests that dynamics are also targeted by HI. Mitochondrial morphology and function is regulated by a cycle of fission and

fusion, coupled with mitophagy (mitochondrial recycling) and biogenesis which maintain mitochondrial integrity and health (Figure 2).

Fission and Fusion

Mitochondria undergo continuous fission and fusion through highly regulated processes in response to metabolic demands, ensuring energy requirements are met [21, 74, 75]. Fission is required for cell division, biogenesis and quality control whereas fusion upregulates ATP production as well as ensuring the mixing of mitochondrial contents [74, 76]. In response to stress, low levels of mitochondrial damage can also be corrected by fusion, creating a larger mitochondrion with greater buffering capacity [74]. It is therefore unsurprising that perturbations in the regulation of fission and fusion are implicated in the development of pathologies including numerous neurodegenerative diseases [77], cancer [78], cardiovascular disease [79], obesity and diabetes [80].

During early postnatal life, there is a substantial metabolic demand in the brain and a significant increase in mitochondrial number is observed. This early period is coupled with neuronal development and the subsequent high demand for energy to maintain homeostasis sensitises the brain to injury following dysfunctional mitochondria (reviewed in [21]). The importance of faithful fission and fusion in neurons cannot be underestimated as defects in fusion result in early onset conditions such as Dominant Optic Atrophy and Charcot Marie Tooth disease type 2A [74].

The processes of fission and fusion are regulated by members of the GTPase Dynamin family. Fission is triggered through Drp1 and fusion through Mfn1/2 (outer mitochondrial membrane fusion) and OPA1 (inner mitochondrial membrane fusion) [74, 81-84]. Both fission and fusion proteins are regulated through proteolysis and posttranslational modifications [74]. Ultimately, an effective fission and fusion cycle allows separation of dysfunctional and functional mitochondria, enabling selective removal by mitophagy, leaving a functional mitochondrial network behind to aid cellular recovery [85].

Fission

Drp1 originates from nuclear DNA and following translation, remains cytosolic [82, 86, 87], undergoing posttranslational modification, including phosphorylation prior to recruitment to the mitochondria [87]. Although contact with the endoplasmic reticulum is believed to designate the fission point on the mitochondrion [88], Drp1 is essential for the fission process, inducing fission via its GTPase activity following self-assembly [89]. Drp1 is maintained in its cytosolic location through scaffolding in a complex with AKAP which facilitates its phosphorylation at Ser 637 by protein kinase A (PKA) and inhibits its GTPase activity [90]. Upon dephosphorylation by calcineurin [91] or PP2A [92] and rephosphorylation at Ser 616 by cdk1, Drp1 translocates to the mitochondrion and binds to one of a

number of OMM-localised receptors including Fis1, Mff, MiD49 and MiD51 [86, 93]. The interaction between one of these membrane-anchored proteins and Drp1 is essential for the fission process, however, the specific mechanism of recruitment is still unclear [87]. Although Fis1 was the first mitochondrial receptor to be identified for Drp1 [94, 95], recent evidence supports Mff as mediating the majority of Drp1 interactions [96]; overexpression of Mff lead to excessive mitochondrial fragmentation [86]. At the mitochondrial membrane, Drp1 spirals around mitochondria forming a helical structure and causing GTP-driven constriction [89] which results in severing of both the outer and inner mitochondrial membranes [21, 74]. Additional regulation of the mitochondrial recruitment of Drp1 was recently identified through its trimolecular interaction with Mff and MiD49/51. High expression levels of MiD49/51 results in suppression of fission, whereas lower levels of MiD49/51 promote fission [86].

Fusion

Fusion is governed by the co-operative action of Mfn1 and Mfn2 at the OMM and OPA1 at the IMM. At the OMM, interactions between Mfn1 & Mfn2 link adjacent mitochondria together and allow GTP-mediated fusion of the outer mitochondrial membrane [97]. Both proteins are required during early development as Mfn1 & Mfn2 single knock-out mice die before birth and the double knock-out mice die mid gestation due to insufficient mitochondrial fusion in the placenta [98]. Both Mfn1 and Mfn2 are capable of regulating OMM fusion, although Mfn1 is more efficient than Mfn2 and is required for efficient OPA1 function [99, 100]. Mfn2 is also reported to be located in the endoplasmic reticulum (ER) membrane and can therefore facilitate ER-mitochondrial interactions, although aspects of this tethering remain unclear [101, 102]. However, the structural and functional similarity between them enables each Mfn to be capable of partially compensating for loss of the other [98].

OPA1 is located on the inner mitochondrial membrane and can exist as eight isoforms in humans, due to alternate splicing of exons 4, 4b and 5 [103, 104]. Efficient IMM fusion requires a combination of long and short forms of OPA1 which are generated through proteolytic cleavage at the ubiquitous S1 site or the variant-dependent S2 site [104, 105]. OPA1 cleavage occurs due to the actions of the ATP-dependent i-AAA-protease Yme1L and the ATP-independent zinc-metalloprotease Oma1 [106, 107]. Yme1L cleaves OPA1 at the S2 site, and the subsequent mix of long, membrane-bound OPA1 and short, soluble OPA1 is optimal for OPA1 function [105, 108]. However, loss of mitochondrial membrane potential results in Oma1-mediated cleavage of OPA1 at the S1 site generating excess short forms of OPA1 and rendering the mitochondrion fusion-incompetent [106, 107].

As mentioned earlier, Bax-mediated MOMP and subsequent cytochrome c release is pivotal in the development of neonatal brain injury in response to HI insult [18, 35, 109, 110]. However, the majority

of cytochrome c is held within the cristae, relying on cristae remodelling to allow it access to move into the intermembrane space once apoptosis is triggered. Not only is OPA1 critical for IMM fusion, but it is also necessary for maintaining stable cristae junctions [111, 112] and for efficient function of the respiratory complexes of the electron transport chain [113]. Inhibition of OPA1 leads to cristae disorganization [114] and a number of studies have highlighted that this cristae remodelling is triggered by Bcl2 family members such as Bax, Bim and t-Bid [112, 115, 116]. A recent study proposes that Bax/Bak oligomerisation at the mitochondrial membrane can act as a trigger for Oma1 activation [117]. The authors also suggest that induction of tBid may also play a role in the activation of Oma1, and speculate that in order for these actions to occur, there needs to be interaction between Bax/Bak oligomers and the contact points for inner and outer mitochondrial membranes.

Mitochondrial dynamics and neonatal brain injury

The impact of HI injury on mitochondrial dynamics in the immature brain has been determined experimentally *in vitro* using oxygen-glucose deprivation (OGD) in primary neurons as well as *in vivo* using a well characterised rodent model of neonatal hypoxic-ischaemic brain injury. *In vitro*, OGD results in a rapid induction of fission in primary neurons [75, 118] coinciding with the pathological cleavage of OPA1 into short fusion-incompetent forms. This degradation of long OPA1 was mirrored *in vivo* at 24h after induction of HI injury [118]. Inhibition of Drp1 expression by siRNA (and thus maintenance of fusion) in hippocampal cells provided protection after OGD and glutamate excitotoxicity [119]. While there are a number of studies examining mitochondrial morphology in the adult brain after ischaemic injury [120], there are currently few *in vivo* studies of the immature brain after HI. However, Demarest and colleagues [121] recently quantified brain mitochondrial size after neonatal HI in immature rats and found that there was a rapid induction of mitochondrial fragmentation. Interestingly, this study identified sex-specific differences with the degree of fission in the injured hemisphere being more marked in males than females [121].

Mitochondrial dynamics are also impacted in response to immune challenge in the immature brain. As well as inducing metabolic reprogramming, LPS treatment (100ng/ml) results in excessive mitochondrial fission in primary microglia [122]. Both the fission phonotype and the LPS-induced metabolic switch can be prevented by treatment with an inhibitor of fission. Therefore, altering mitochondrial dynamics can be a therapeutic modality for preventing neuroinflammation-induced over-activation of microglia thereby ameliorating neuronal or oligodendroglial death following perinatal inflammation.

Mitophagy - mitochondrial quality control

Mitochondrial mitophagy is defined as the selective degradation (or selective autophagy) of sufficiently damaged mitochondria through an autophagosomal-lysosomal pathway [123]. The most well characterised mechanism triggering mitophagy relies on PTEN-induced putative kinase 1 (PINK1) / Parkin [4, 5]. The importance of mitophagy is underlined by the availability of additional pathways to mitochondrial recycling including the BNIP3, NIX and FUNDC1 ([124]; Figure 3). Once mitophagy is triggered, mitochondrial-localised receptors bind LC3B (Figure 3), a microtubule-associated protein converted from LC3A through conjugation with phophatidylethanolamine. This conversion allows LC3B to relocate from the cytosol into phagophore membranes thus linking the damaged mitochondrion with the vesicle mediating its degradation (Figure 2, [125]).

PINK1 and Parkin

PINK1 is a serine/threonine kinase targeted to mitochondria via its N terminal signal sequence. It is imported into the IMM in healthy mitochondria in the presence of a high mitochondrial membrane potential ($\Delta\Psi_m$) [124, 126]. However, once there, PINK1 is degraded by matrix processing peptidase (MPP) and presenilin-associated rhomboid-like (PARL)[124]. Conversely, upon cellular insult resulting in mitochondrial depolarisation, import of PINK1 to the inner mitochondrial membrane is inhibited and it subsequently accumulates on the outer mitochondrial membrane [126]. PINK1 then phosphorylates ubiquitin (at Ser 65) resident on OMM proteins [127, 128], recruiting the E3 ubiquitin ligase Parkin to bind to the phospho-ubiquitin [129]. As Parkin itself contains a ubiquitin-like domain, it becomes a substrate for PINK1 phosphorylation, enabling an increased, Parkin-mediated ligation of ubiquitin to proteins on the OMM, thus increasing substrate availability for PINK1 [130]. Adaptor proteins (SQSTM/p62, optineurin, NDP-52 *etc.;* [131]; Figure 3A) are recruited which lead to binding of LC3B and engulfment of the dysfunctional mitochondrion by an autophagosome which, when fused with a lysosome, promotes degradation of its contents. This mechanism allows for the selective degradation of a damaged mitochondrion and impairment is thought to play a key role in Parkinson's disease as well as ageing in general. It is worth highlighting that mechanisms of PINK1/Parkin mitophagy have largely been elucidated *in vitro* and utilising mitochondrial toxins targeting large populations of mitochondria in order to instigate mitophagy. There still remains a number of questions about mechanisms triggered *in vivo* in response to discrete mitochondrial dysfunction [132].

BNIP3 and NIX

Other PINK1-independent mechanisms of mitophagy have also been identified. Bcl-2 and adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L), also known as NIX, are single pass transmembrane receptors localised to the outer mitochondrial membrane which are capable of recruiting LC3B (Figure 3B). As BNIP3 expression is regulated by HIF1-α expression, it becomes

activated in response to hypoxia and has been implicated in mitophagy in response to stroke [133]. Both BNIP3 and NIX are implicated in Bax-mediated cell death with hypoxia-activated Bnip3 and excitotoxicity-activated Nix resulting in Bax and Bak signalling [134, 135]. BNIP3 also links fission and mitophagy as there is a requirement for Drp1 (as well as Parkin) for mitophagy in cardiomyocytes [136].

FUNDC1

FUNDC1 is mitochondrial outer membrane protein, which binds LC3B directly in response to hypoxia (Figure 3C; [137, 138]). FUNDC1 and LC3B becomes stabilised under hypoxic conditions, which is attributed to dephosphorylation of FUNDC1 at Tyr18 during hypoxia. Indeed, Src kinase inhibits FUNDC1-mediated mitophagy in response to hypoxia [137]. FUNDC1 is also negatively regulated by ser13 phosphorylation by casein kinase 2 which can be counteracted by the action of PGAM5-mediated dephosphorylation in response to a hypoxia cue [139]. Conversely, PGAM5 can be inhibited by interaction with Bcl-xL (but not Bcl-2) thus providing a regulatory signalling loop dependent on the cellular stress [140].

Mitophagy and neonatal brain injury

The role of mitophagy in the development of neonatal brain injury after HI is still unclear and it remains to be determined whether induction of mitophagy post injury would be beneficial or deleterious. Current evidence is circumstantial as it is based mainly on modulating components of the autophagy pathway rather than mitophagy directly. For example, in an *in vitro* hippocampal slice culture model of immature brain, cell death as a result of exposure to OGD was significantly reduced when the slices were pre-treated with a pharmacological inhibitor of autophagy, 3-methyladenine (3-MA; [141]). *In vivo*, 3-MA inhibition prior to HI prevented increased LC3B expression in neonatal rats as well as reducing memory impairment in subsequent behavioural tests [142]. In line with this observation, neonatal HI-induced brain injury in mice lacking a protein critical for autophagosome formation (*Atg7*$^{-/-}$) was significantly reduced compared with WT litter mates [143]. However, more specifically, BNIP3 was shown to co-localise with LC3B in mice after neonatal HI *in vivo* and OGD *in vivo*, and infarct volume and cell death were prevented when BNIP3 expression was genetically ablated [133]. *Bnip3* gene expression was also upregulated in neonatal rat brain in a study examining the effects of hypoxic preconditioning, a sublethal insult which is known to induce subsequent neuroprotection after HI injury [144]. Mitochondrial and lysosomal co-localisation were also observed in immature rat brain but in this study there were marked sex-specific differences, with greater induction of mitophagy in females versus males [121] echoing previous observations of increased basal autophagy and upregulated LC3B expression in female rats after neonatal HI [145]. As brain injury in males was more

severe, these data contradict studies mentioned earlier which suggested that inhibition of autophagy/ mitophagy may confer protection.

Biogenesis

Biogenesis is required for the increase in mitochondrial mass within a cell and is dependent on both fission of existing mitochondria followed by a co-ordinated programme of transcription and translation principally regulated by the transcription factor PGC-1α [146]. This process is crucial to allow for growth and recovery of the existing mitochondrial network following depletion by mitophagy [147]. Ultimately, this process ensures the mitochondrial network is able to efficiently support and adapt to the metabolic needs of the cell [147]. The whole process is carefully orchestrated, involving synthesis driven by both mitochondrial and nuclear DNA and coordinated import of around 1000 lipids and proteins [21, 146, 147].

PGC-1α is widely considered the master regulator and is often upregulated during times when the demand to energy is increased [147, 148]. PGC-1α is often expressed at high levels within neurons and due to their inherent high demand for energy [146, 148]. PCG-1α is unable to bind DNA directly, instead forming complexes with other transcriptional factors (NRF1/2, PPAR, ERRs) to drive transcription of nuclear genes encoding mitochondrial proteins [21, 146, 147]. NRF1 and NRF2 drive TFAM expression which is expressed from nuclear DNA and is transported into mitochondria, driving transcription of 13 key enzymes from mtDNA needed for oxidative phosphorylation [21, 146, 147]. PPARs and ERRs mainly drive transcription of key proteins from nuclear DNA involved in oxidative phosphorylation [147].

During development, mitochondria undergo rapid biogenesis, coupled with high expression of PGC-1α [21] and PGC-1α knockdown mice showing evidence of neuropathology and abnormal behaviour whilst *in vitro*, neurons show development changes following manipulation of PGC-1α [146]. Central nervous system neurons are derived from proliferative neural stem cells by neurogenesis, newly formed neurons grow rapidly, forming synapses and the future neuronal circuits [148]. During such times mitochondria biogenesis is essential for production of the large amount of ATP required; however, there is further evidence to suggest the presence and location of such mitochondria during development play a regulatory role in designing the cytoarchitecture [148]. PGC-1α has also been shown to play a role in formation and maintenance of synapses during development [148]. Such a role may be linked to neuronal requirement of an increase in energy during these processes, suggesting biogenesis plays an important role in development [148].

Biogenesis and neonatal brain injury

Alterations in mitochondrial biogenesis may be predicted as the prerequisite fission appears to be upregulated immediately after neonatal HI [121]. *In vitro*, cerebellar granule neurons (CGNs) isolated from female neonatal mice were reported to have reduced mitochondrial DNA content, reduced expression of transcription factors required for mitochondrial biogenesis and reduced membrane potential and ATP. Therefore in contrast with enhanced protection of females suggested in studies of mitophagy, female CGNs were more susceptible to cell death [149]. *In vivo*, neonatal HI injury induces a marked upregulation of mitochondrial DNA mass and key transcription factors, which reaches a peak at 24h post injury [150].

Mitotherapeutics – a new avenue for treating neonatal brain injury

Given the variety of ways in which mitochondria are targeted after exposure to hypoxic-ischaemic injury, it is logical to explore therapies which maintain mitochondrial health, to act in synergy with therapeutic hypothermia. A number of therapies which impact on mitochondrial function are already approved for clinical trial (erythropoietin, melatonin, stem cells) and have been reviewed very recently as well as a number of additional suggestions of mitochondrial protein targets ([146]; table 1). However there are an increasing number of small molecule activators and inhibitors which are targeted directly to the mitochondrion which may prove invaluable as therapeutic tools (table 1). Selective targeting to the mitochondrion can be achieved using lipophilic cation conjugation such as tetra- and triphenylphosphonium (TPP) as well as mitochondria-penetrating peptides which can be delivered orally, intravenously, intraperitoneally or intranasally [151, 152]. However, it must be noted that TPP delivery is not as efficient at delivery to the brain as to peripheral organs [152]. Other delivery systems have been developed (gold, titanium nanoparticles etc.) which aim to circumvent disadvantages of liposome mediated systems such as size limitations [153]. Targeting antioxidants and bioactive therapeutics selectively to mitochondria is rapidly becoming an achievable goal for development of adjunct therapies.

Direct modulators of mitochondrial function

Coenzyme Q10 (CoQ10)

acts as an electron acceptor to reduce the production of ROS by complex I. CoQ10 administration post injury was found to be neuroprotective, reducing the effects of ischaemia in a wide variety of rodent models including traumatic brain injury [154], middle cerebral artery occlusion (MCAO; [155, 156]), MCAO with hyperglycaemia [157], ischaemia in aged rodent models of

Alzheimer's disease [158]. Currently there are no studies of CoQ10 in neonatal hypoxic-ischaemic brain injury models.

Mitoquinone (MitoQ) is also a ubiquinone derivative conjugated to TPP which acts as an anti-oxidant to prevent the production of superoxide and further ROS generation. In inflammation models, MitoQ has been shown to suppress the production of IL-6, TNF, CXCL8/IL-8, and IL-10 in peripheral blood monocytes from patients with TNF-Receptor Associated Periodic Fever Syndrome (TRAPS) offering potential therapeutic benefit [159]. Although promising in ischaemia-reperfusion studies in peripheral tissues e.g. heart [160], there are limited data regarding MitoQ post-treatment in hypoxic-ischaemic brain injury [161]. The limited data on neonatal brain HI suggests that mitoQ is not neuroprotective in medium spiny neurons of the thalamus [162]. However studies in adult ischaemic injury suggests that intranasal delivery of plant-derived ubiquinones, plastoquinone and thymoquinone conjugated to rhodamine 19, may have significant neuroprotective properties post injury [152].

Metformin is an inhibitor of mitochondrial respiration with actions on mitochondrial complex I [163, 164]. It was recently administered after neonatal HI in rats and was found to improve performance in behavioural tests as well as promote oligodendrocyte survival with consequent effects on remyelination [165]. Metformin has been successfully used in rodent models to target inflammatory T cells in lupus in combination with glycolytic inhibition using 2-deoxyglucose. Metformin is also used to inhibit interleukin 1β (IL1β) production in LPS-induced sepsis [64] and maternal metformin treatment in a rat model of metabolic syndrome is beneficial in preventing foetal inflammation, a risk factor for perinatal HI [166].

S1/S3QELs The mitochondria targeted antioxidant S1QELs (site IQ electron leak) and S3QELs (site IIIQ electron leak) are novel compounds recently developed as selective suppressors of complex I-dependent and complex III-dependent $O_2^{\cdot -}$ /H_2O_2 production which do not inhibit oxidative phosphorylation [167]. These have currently not been tested *in vivo* although promising results have been obtained after hypoxia *in vitro* [168] and in ischaemic reperfusion injury in perfused mouse heart [169].

Daidzein (also Daidzein sulphonate sodium) is a plant-derived polyphenol which was first reported as protective in cancers and inflammatory conditions. However recent studies suggest that Daidzein has anti-oxidant effects in rodent models of MCAO preventing ROS production, mitochondrial swelling and increasing antioxidant activities (superoxide dismutase, glutathione peroxidase [170-173]). Neither daidzein nor its derivatives have been tested in neonatal HI brain injury.

mDivi-1 was originally identified as a specific inhibitor of the fission mediator Drp1. However, a very recent study suggested that mdivi-1 is a poor inhibitor of Drp1 and rather acts as a reversible mitochondrial complex I inhibitor and regulator of ROS production [174]. Regardless, a number of studies have shown that mdivi-1 improves outcome after OGD [119, 175-178] and abrogates brain injury after MCAO [175], subarachnoid haemorrhage [179], transient global ischaemia [180] and traumatic brain injury [181].

P110 is a peptide derived from the interaction site of Drp1 and Fis1 and acts to block the localisation of Drp1 to the mitochondrion [182]. P110 provided neuroprotection in cell models of Parkinson's disease [182] and Huntington's disease [183] dopaminergic neurons as well as in rodent models of neurodegeneration [183, 184] and myocardial infarction [185]. It has not yet been tested in neonatal HI brain injury.

Conclusions and Perspectives

The urgent unmet clinical need to provide synergistic neuroprotection alongside therapeutic hypothermia for neonates suffering the consequences of birth asphyxia requires novel avenues of drug discovery to be explored. Therapeutics which have achieved preclinical success in models of adult brain hypoxic-ischaemic injury cannot simply be transferred to the neonate. For a number of years we have understood that mitochondria act as the target of the injury response, and the pathway in which MOMP in the immature brain leads to apoptosis and necroptosis during the secondary phase of injury has been well characterised. Pharmacological tools developed from this research (e.g. the caspase-2/3 inhibitor TRP601 [47]) have already provided proof-of-concept data suggesting that intervention post injury can be efficacious. As we come to understand more about fission, fusion, mitophagy and biogenesis of the mitochondrion and how this lifecycle becomes adversely affected by HI injury, we are provided with additional therapeutic targets. Emerging data regarding sex-specific differences in fission, mitophagy and biogenesis responses require us to be rigorous in preclinical testing of new therapeutics targeting these points. Direct targeting of mitochondria is now becoming a reality and a wealth of small molecule modulators offers the potential sensitivity and specificity required to ameliorate the lifelong impact of hypoxic ischaemic brain injury in these vulnerable infants.

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Table 1: Mitochondrial therapeutics with potential to ameliorate neonatal HI brain injury

	Agent	Model	Mitochondrial Action	Reference
Human	Erythropoietin (EPO)	Human clinical trial for neonatal HIE and preterm brain injury	Anti-inflammatory Promotes mitochondrial biogenesis via PGC1α upregulation	[186, 187]
	Melatonin	Human clinical trial for neonatal HIE	Mitochondrial Antioxidant, decreases fission, regulates mitophagy	[188, 189]
	Umbilical Cord Blood Cells (UCBCs)	Human clinical trial of UCBCs and EPO for Cerebral Palsy	Anti-apoptotic, anti-inflammatory, mitochondrial transfer via tunnelling nanotubes	[190, 191]
Rodent	Coenzyme Q10	Traumatic Brain Injury, MCAO ± hyperglycaemia, ischaemic injury in aged animals	Anti-oxidant, acts as an alternative electron acceptor to prevent superoxide formation	[154-158]
	Mitoquinone	Ischaemia/reperfusion in heart	Anti-oxidant, acts as an alternative electron acceptor to prevent superoxide formation	[160]
	Metformin	Neonatal HI, Lupus, maternal metabolic syndrome (targeting foetal inflammation)	Mitochondrial complex I and mitochondrial respiration	[64, 165, 166]
	S1/S3QELs	Ischaemia reperfusion injury in perfused heart	Anti-oxidant, preventing electron leakage at complex I and III	[167, 169]
	Daidzein	MCAO	Anti-oxidant, prevents ROS accumulation, mitochondrial swelling and promotes antioxidant enzyme activities	[170-173]
	mDivi-1	MCAO, subarachnoid haemorrhage, transient global ischaemia, traumatic brain injury	Reversible mitochondrial complex I inhibitor, regulating ROS production	[174, 175, 179-181]
	P110	Neurodegeneration (PD, HD), Myocardial Infarction	Fission inhibitor (Drp1:Fis1 interaction)	[182-185]

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Figure Legends

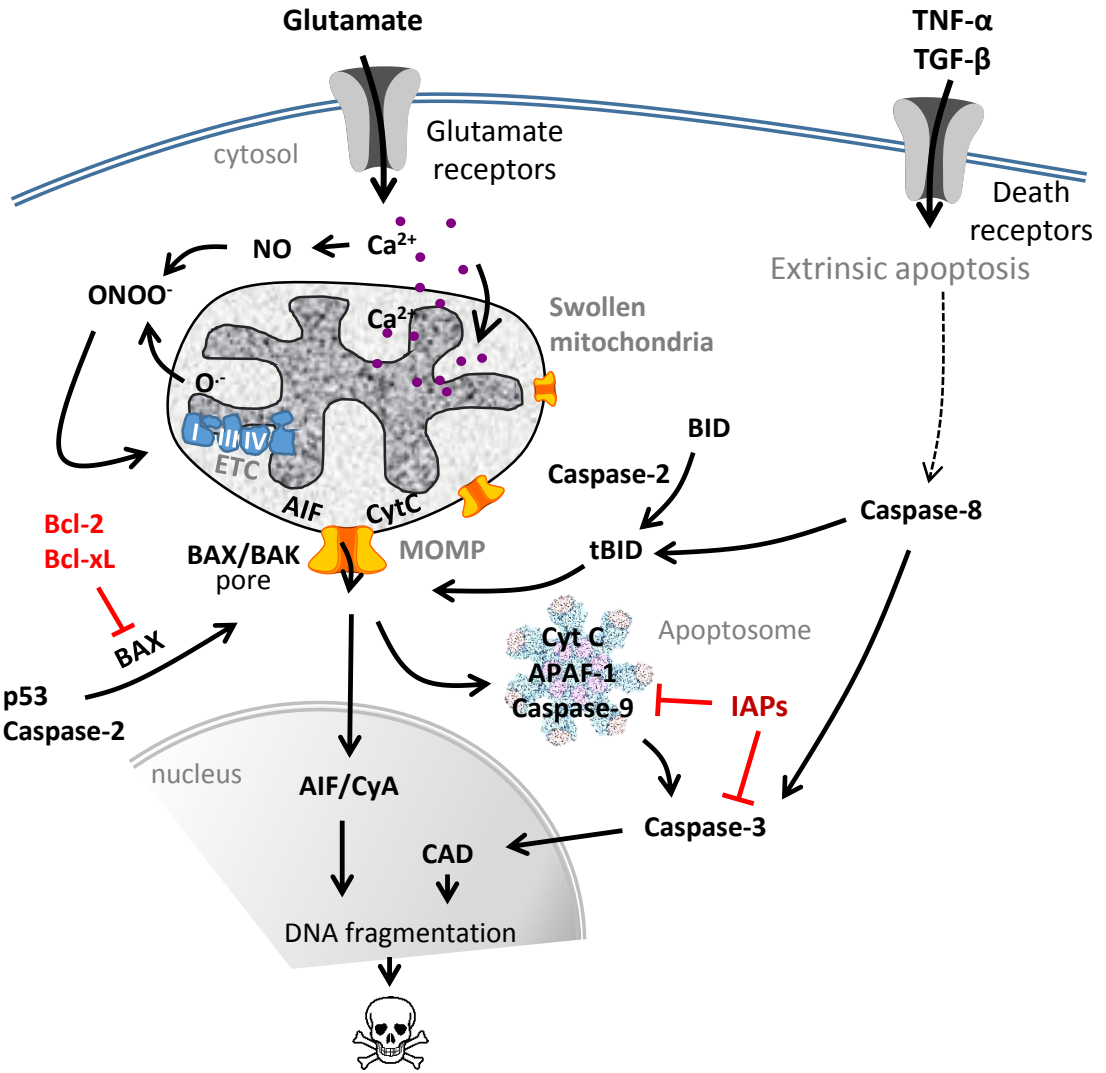
Figure 1: Neonatal HI injury results in mitochondrial outer membrane permeabilisation (MOMP) and cell death. Neonatal HI induces mitochondrial accumulation of calcium, increased production of reactive oxygen and reactive nitrogen species. Changes in Bcl-2 family proteins induce Bax-dependent MOMP leading to the release of cytochrome c (cyt c) and apoptosis-inducing factor (AIF). The apoptosome is formed of cyt c, APAF-1 and caspase-9 which leads to caspase-3 activation, caspase-activated DNases (CAD) and DNA degradation. AIF forms a complex with cyclophilin A (CyA) which translocates to the nucleus and induces chromatinolysis and apoptotic cell death. B) Concomitantly,

inflammatory microglia and astroglia will release death receptor ligands (FasL, TWEAK, TRAIL, LPS) leading to the activation of death receptors, which in turn contribute to the induction of apoptosis.

Figure 2: Mitochondrial dynamics, mitophagy and biogenesis. Fission, fusion, mitophagy and biogenesis are balanced to ensure efficient ATP production through fusion (with minimal generation of ROS/RNS) and degradation of damaged mitochondria (through fission and mitophagy). Biogenesis restores fissioned mitochondria lost to mitophagy. During mitophagy, damaged mitochondria are recruited to the phagophore through LC3B and engulfed into the autophagosome. Fusion with a lysosome results in degradation of the mitochondrion within the autolysosome. Key proteins for each stage are shown together with the regulation of Drp1; Ser 637 phosphorylation keeps Drp1 in the cytosol, whereas dephosphorylation by calcineurin or PP2A and rephosphorylation at Ser616 results in its mitochondrial location.

Figure 3: Mechanisms of mitophagy. A) On mitochondrial depolarisation, PINK1 degradation is prevented. PINK1 thus accumulates at the OMM where it phosphorylates ubiquitin present on proteins of the OMM. To amplify this response, PINK1 also phosphorylates the ubiquitin-like domain of E3 ubiquitin ligase Parkin, resulting in its migration to the mitochondrion and its recruitment of ubiquitin, providing more substrates for PINK1 phosphorylation. Phosphorylated Ub acts to recruit autophagy receptors such as OPTN, NDP52 and p62/SQSTM which bind to phagophore-located LC3B and enable the dysfunctional mitochondria to be engulfed into an autophagosome where it is subsequently degraded on fusion with a lysosome. **B)** BNIP3 and Nix are mitochondria-located transmembrane proteins stabilised in response to hypoxia. Both are capable of binding LC3B directly and recruiting the mitochondrion to the autophagosome. **C)** Dephosphorylation of FUNDC1 by PGAM5 in response to hypoxia reveals its canonical LC3B binding site through which it can bind LC3B directly and recruit the phagophore membrane, enabling mitophagy.

Figure 1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TNF-α
TGF-β
cytosol
Glutamate receptors
Death receptors
Extrinsic apoptosis
NO
Ca2+
ONOO-
Swollen mitochondria
O2-
I II III IV
ETC
AIF
CytC
BID
Caspase-2
Caspase-8
Bcl-2
Bcl-xL
BAX/BAK pore
MOMP
tBID
BAX
p53
Caspase-2
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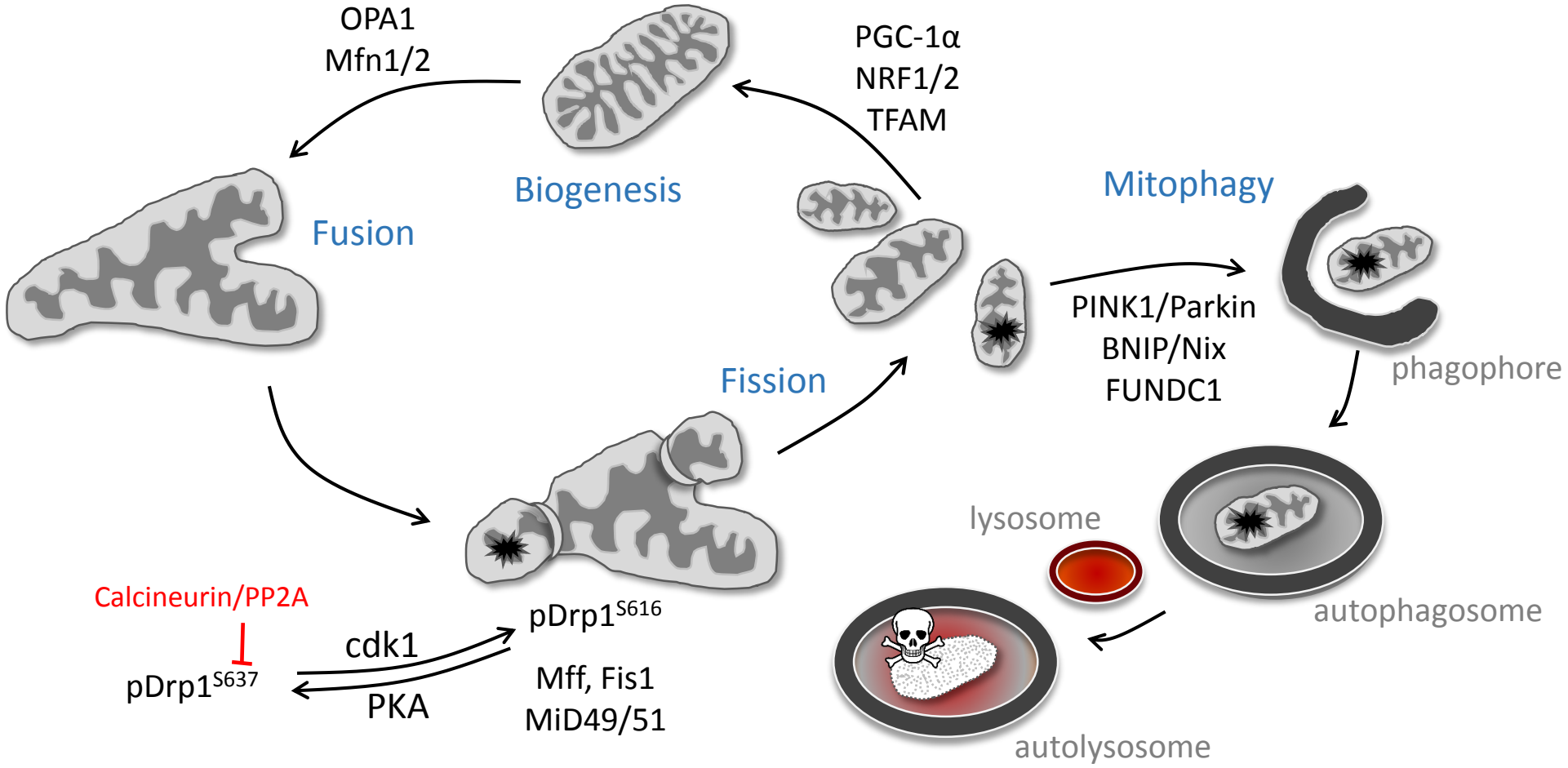
Apoptosome
IAPs
nucleus
AIF/CyA
Caspase-3
CAD
DNA fragmentation

Figure 2


OPA1
Mfn1/2
PGC-1α
NRF1/2
TFAM
Biogenesis
Fusion
Mitophagy
PINK1/Parkin
BNIP/Nix
FUNDC1
phagophore
Fission
lysosome
autophagosome
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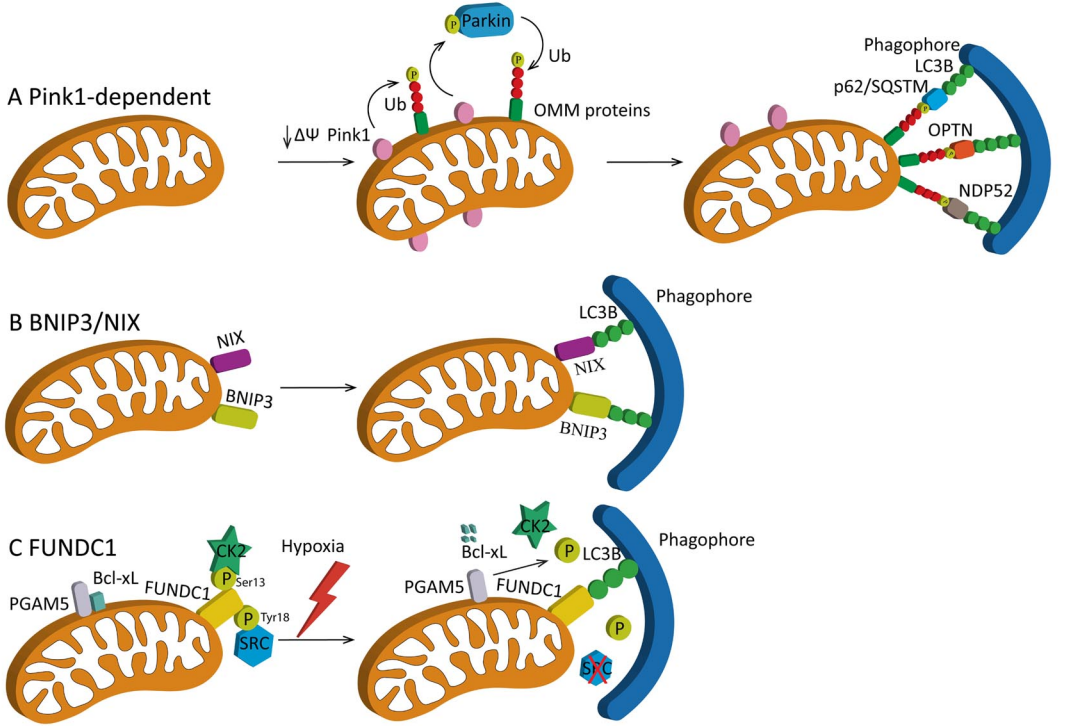
A Pink1-dependent
Parkin
Ub
Ub
OMM proteins
↓ΔΨ Pink1
Phagophore
LC3B
p62/SQSTM
OPTN
NDP52
B BNIP3/NIX
NIX
BNIP3
LC3B
Phagophore
NIX
BNIP3
C FUNDC1
CK2
Hypoxia
PGAM5
Bcl-xL
FUNDC1
Ser13
Tyr18
SRC
Bcl-xL
CK2
LC3B
Phagophore
PGAM5
FUNDC1
SRC